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LETTER

Comment on "Effectiveness of antimicrobial photodynamic therapy (AmPDT) on *Staphylococcus aureus* using phenothiazine compound with red laser"

Joanna Nakonieczna[1]

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After reading the following paper: "Effectiveness of antimicrobial photodynamic therapy (AmPDT) on *Staphylococcus aureus* using phenothiazine compound with red laser" Monteiro, J.S.C., de Oliveira, S.C.P.S., Pires Santos, G.M. et al. Lasers Med Sci (2016). doi:10.1007/s10103-016-2079-4, I would like to make a comment as to the method of calculating AmPDT treated bacteria. Phenothiazinium photosensitizers have been studied in the field of antimicrobial photodynamic research for years [1–4]. The most frequently studied Methylene Blue and Toluidine Blue showed light-enhanced antimicrobial efficacy towards Gram-positive and Gram-negative species. To properly assess the effectiveness of antimicrobial compound, calculating the $\log_{10}$ reduction from the initial number of CFU/ml are typically performed. Based on general guidelines of the American Society for Microbiology reduction the number of bacteria at least 3 $\log_{10}$ CFU/ml is considered biologically relevant [5]. In the paper by Monteiro et al., only 2 $\log_{10}$ reduction was observed, which is not considered antibacterial action.

The data in the Figure 1, Figure 2, and Figure 3 shows that the authors used c.a. 10^2 CFU/ml in all their AmPDT experiments. First, it is inconsistent with the statement given in the section Material and Methods, where the authors stated: "The logarithm of CFU/mL ($\log_{10}$ CFU/mL) was calculated…" and also "For the quantification of colony-forming units (CFU), the suspension was standardized (…) to approximate number 3×10^8 CFU/mL." Subsequently, 10 μL of this suspension was inoculated in 1 mL of TSB. This finally gives approx. 10^6 CFU/ml. Secondly, the presented data showed that the reduction in bacterial cell survival was at most 2 $\log_{10}$ units, which further means that the decrease in survival was at most 99%, not 100%. It is commonly known that AmPDT strongly depends on cell density. The PDI efficacy decreases dramatically with increase of cell density. This was observed also for *S. aureus* and Toluidine Blue [6]. Thus, using such a low concentration of bacteria (10^2 CFU/ml), it is highly unlikely to make proper translation from in vitro to in vivo studies. The parameter of cell density used in the experiments should be very carefully taken into consideration. Guidelines for performing bactericidal tests that were published in 1999 by the NCCLS [7] indicate critical methodology components for minimal bacteriocidal concentration as inoculum of $\geqslant 5 \times 10^5$ CFU/mL and a subculture volume of 0.1 ml to accurately predict whether ⩾99.9% of the bacteria were killed. In a typical AmPDT experiment, even higher number of cells are analysed (e.g. 10^6–10^8 CFU/ml). Bactericidal tests must be performed with a sufficient inoculum and subculture volume to ensure accurate determination of the 99.9% killing endpoint [5]. Inoculum size and subculture volume are also critical to studies of combinations of antimicrobial agents [5]. In my opinion, this is a serious limitation of the presented study by Monteiro et al.

✉ Joanna Nakonieczna
joanna.nakonieczna@biotech.ug.edu.pl

[1] Laboratory of Molecular Diagnostics, Department of Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Abrahama 58, 80-307 Gdansk, Poland

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